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Research Article

Response of Maize Seedlings to Cadmium Application after Different Time Intervals

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Present study was conducted to appraise the inhibitory effects of cadmium applied at different time intervals on various growth and biochemical parameters in two maize lines, Maize-TargetedMutagenesis 1 and 2 (MTM-1 and MTM-2). Twenty-day-old seedlings were exposed to 0, 3, 6, 9, and 12 mg $CdCl_2$ kg^{-1} sand. Both maize lines exhibited significant perturbations in important biochemical attributes being employed for screening the crops for cadmium tolerance. The results showed that a higher concentration of cadmium (12 mg $CdCl_2$ kg^{-1}) considerably reduced the plant growth in line MTM-1 on the 5th, 10th, and 15th day after the treatment. In contrast, irrespective of exposure time, the plant biomass and leaf area did not show inhibitory effects of cadmium, specifically at 3 mg $CdCl_2$ kg^{-1} in line MTM-2. In addition, MTM-2 was found to be more tolerant than line MTM-1 in terms of lower levels of hydrogen peroxide (H2O2), malondialdehyde (MDA) contents, and relative membrane permeability (RMP). Moreover, H2O2, MDA, RMP, and anthocyanin increased at all levels of cadmium in both lines, but a significant decline was observed in photosynthetic pigments, total free amino acids, and proline contents in all treatments particularly on the 10th and 15th day after treatment.

1. Introduction

Heavy metal stress is one of the major abiotic stresses that cause environmental pollution in recent decades. Cadmium is a toxic pollutant that negatively affects the plant growth. Cadmium is added to the environment by different sources and is persistent for a long time in the environment; it comes into the food chain through plants and threatened the ecosystems [1]. Cadmium is taken up by the plant roots and loaded into the leaves through the phloem and can be accumulated in all parts of the plants [2]. Thus, instead of just reducing the crop productivity and quality [3], it causes a severe health risk to mammals and humans [4].

The cadmium affects the whole life cycle of plants. It inhibits the seed germination [5], disturbs the photosynthetic metabolism and transpiration rate, reduces enzymatic and non enzymatic activities [6], disturbs water homeostasis and ionic relations [7], mineral nutrition [8], induces synthesis of reactive oxygen species, and strongly reduced the biomass production [8]. The cadmium stress causes chlorosis and leaf and root necrosis resulting in stunted growth in the majority of the plants [8]. However, the amount of cadmium deposited into the root, shoot, and interveins of leaves considerably differed among different species [9].

Cadmium induced oxidative stress at the cellular level in different plants [10]. Moreover, cadmium triggers accumulation and/or synthesis of reactive oxygen species like superoxide radical, singlet oxygen, hydrogen peroxides, and hydroxyl radicals [11] that may cause cell death by lipid peroxidation, oxidation of proteins [12], damaging DNA [13], and affect the activities of antioxidant enzymes (superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase, catalase, and glutathione reductase) involved in the oxidative defense system [14]. Thus, a balance between the production of activated oxygen species and quenching activity of antioxidant was disturbed [15]. To repair the inhibitory effects

of reactive oxygen species, plants make use of antioxidant defense machinery including enzymatic and nonenzymatic defense system [16].

Maize (*Zea mays* L.) is a suitable crop for tropics and subtropical regions of the world. Maize can be an excellent model plant to study the physiological changes responsible for reduced productivity under stressful conditions. Being a rich source of nutrition (72% starch, 10% protein, 8.5% fiber, and 4.8% edible oil), maize is a major source of food, sugar, cooking oil, and animal feed all over the globe [17].

Keeping the value of maize crop in mind, cadmium stress being the harmful menace to its crop growing, the experiment was performed to assess the effect of various cadmium concentrations on different agronomical, physiochemical attributes of maize seedlings after different time intervals.

2. Materials and Methods

2.1. Plant Material, Treatment, and Plant Growth Conditions. Seeds of two maize lines, Maize-Targeted Mutagenesis 1 and 2 (MTM-1 and MTM-2), were obtained from the Chinese Academy of Agriculture Sciences China and Plant Genetics Resources Institute (PGRI), NARC, Islamabad, Pakistan (Collection Center). An experiment was conducted at the Department of Botany, GC University, Faisalabad, Pakistan. The seeds were disinfected with 0.1% $HgCl_2$ solution for 15 min and washed with distilled water before sowing in the plastic pots containing 10 kg sand. Ten seeds were sown in each pot. The plants were given half strength Hoagland's nutrient solution on a five day basis. After germination, five uniform healthy seedlings were retained in each pot. Twenty-day-old seedlings were exposed to five different levels of cadmium (0.0, 3, 6, 9, and 12 mg $CdCl_2\,kg^{-1}$ sand) in half strength Hoagland's nutrient solution. The experimental design was completely randomized with three replications per treatment. The sampling was done on the 5th, 10th, and 15th day after cadmium treatment.

2.2. Growth Determinations. Leaf surface area was measured using a leaf area meter. After drying in an oven at 70°C for about 72 h, shoot and root dry weights were recorded. The other plant samples were preserved in cooling chamber at −20°C.

2.3. Chlorophyll a, b and Total Carotenoids Contents. Chlorophyll (Chl) a and b and total carotenoids contents were determined after homogenizing fresh leaves (0.1 g) in 80% acetone (10 mL) and centrifuging at 3000 ×g for 15 min. The absorbance from supernatant was determined at 480 nm, 645 nm, and 663 nm using a spectrophotometer (Hitachi U-2001, Tokyo, Japan). The amounts of chlorophyll contents and total carotenoids were calculated as described by Yoshida et al. [18] and Davies [19].

2.4. Relative Membrane Permeability (RMP). Leaf tissues were collected in the test tubes containing distilled water (10 mL), vortexed for 5 s, and the value of EC_0 was measured. Then EC_1 of the filtrate was measured after 24 h by keeping them at 4°C. The filtrate was autoclaved for 15 min for measuring EC_2. The percentage of ions leakage was calculated from the equation of Yang et al. [20].

2.5. Hydrogen Peroxide (H2O2) Contents. These were estimated using the method of Velikova et al. [21]. Leaf tissues (0.1 g) were homogenized with 1 mL of 0.1% (w/v) trichloroacetic acid (TCA) on an ice bath. Then homogenate was centrifuged at 12,000 ×g for 15 min. The reaction mixture consisted of supernatant (0.5 mL), 0.5 mL potassium phosphate buffer (10 mM; pH 7.0), and 1 mL KI (1 M), then vortexed, and the absorbance was measured at 390 nm, while 0.1% TCA used as blank. The H2O2 content was determined from a standard curve, and the values are expressed as μmol g^{-1} fresh weight.

2.6. Malondialdehyde (MDA) Contents. The MDA was assayed according to the method of Heath and Packer [22]. Fresh leaf tissues (0.1 g) were homogenized in 5% (w/v) TCA (1 mL). The homogenate was centrifuged at 12,000 ×g for 15 min. After centrifugation, 1 mL of an aliquot of the supernatant was mixed with 20% TCA (1 mL) containing 0.5% (w/v) thiobarbituric acid. The mixture was warmed at 95°C for 30 min, cooled on ice for a while, and then centrifuged at 7500 ×g for 5 min. The absorbance was recorded at 532 nm and 600 nm, whilst 5% TCA used as blank. MDA contents were calculated using an extinction coefficient of 155,000 nmol mol^{-1}:

$$\mathrm{MDA}\left(\mathrm{nmol\,mL^{-1}}\right) = \left[\frac{\left(A_{532} - A_{600}\right)}{155000}\right] 10^6. \quad (1)$$

2.7. Free Proline Contents. Free proline was determined using the method of Bates et al. [23] Leaf tissue (0.1 g) was homogenized in 3% of aqueous sulphosalicylic acid (5 mL). Filtrate (1 mL) was mixed with acid ninhydrin (1 mL) and glacial acetic acid (1 mL) in a test tube. The mixture was cooled after heating for 10 min at 100°C in an ice bath. The mixture was extracted with toluene (4 mL) and vortexed for 20 s and cooled. The absorbance was measured at 520 nm. The amount of free proline was calculated from the standard curve at 520 nm and expressed as μmol g^{-1} fresh weight.

2.8. Total Free Amino Acid Contents . Total free amino acids were determined by using the ninhydrin method of Hamilton and Slyke [24]. 1.0 g of fresh plant material was extracted by using phosphate buffer (pH 7.0). In 25 mL test tube, 1 mL extract was taken, then 1 mL of 10% pyridine and 1 mL of 2% ninhydrin solution was added in each test tube. And test tubes were heated in a water bath for 30 minutes. The volume was maintained up to 50 mL by using distilled water in each tube. Optical density was measured at 570 nm by using UV-VIS spectrophotometer (Hitachi U-2910, Tokyo, Japan). The amount of total free amino acids was calculated from the standard curve of Lucine at 570 nm and expressed as mg g^{-1} fresh weight.

2.9. Total Anthocyanin Contents. Total anthocyanin contents were determined by the method of Hodges and Nozzolillo [25]. Fresh leaves (0.1 g) of sample were crushed in acidified methanol (2 mL) with the help of pestle and mortar. Then materials transferred to the centrifuge tubes, and heated them in water bath at 50°C for one hour. Centrifuge the materials at 12000 ×g in the centrifuge machine for 15 minutes. The absorbance was measured at 540 nm and 600 nm by using UV-VIS spectrophotometer (Hitachi U-2910, Tokyo, Japan). The amount of total anthocyanin contents was calculated in the original sample at 520 nm and 600 nm, expressed as mgL^{-1} fresh leaves.

2.10. Statistical Analysis. The data collected was subjected to analysis of variance technique (ANOVA) by using a computer software CoStat version 6.2, CoHort Software, 2003 (Monterey, CA, USA). The least significant difference among means was computed. The data in the tables is represented as means ± SE ($n = 3$) for each parameter.

3. Results and Discussion

Cadmium stress significantly ($P \leq 0.001$) decreased different growth attributes of both maize lines (Tables 1 and 2). The data showed that MTM-1 line exhibited markedly lowest growth rate in terms of root and shoot dry weights than MTM-2 at all cadmium levels on the 5th, 10th, and 15th day of sampling. Increasing cadmium levels up to 3 mg $CdCl_2$ kg^{-1} increased shoot and root dry weights and leaf area in MTM-2 than MTM-1 line, while a decrease was noted afterwards on the 5th, 10th, and 15th day of sampling.

Although cadmium toxicity depends upon the type of species and the plant growth stage, it has been shown to reduce crop productivity and quality severely [14]. In the present study, results indicated the differential responses of maize lines to cadmium stress at different intervals of time. Earlier studies have shown that higher level of cadmium changed the pattern of growth and inhibited the plant growth significantly in cucumber at seedling stage [26]. Even at low levels, cadmium is reported to modify the plant metabolism [27]. Changes in plant metabolism could affect the growth patterns as have been reported in some other crops like mung bean [28]. Upon exposure to cadmium, it appeared to inhibit the cell division and thus disrupted root cell expansion and enlargement. In addition, a decreased carbohydrate synthesis due to the inhibitory effect of cadmium on carbohydrate metabolism has also been shown to inhibit shoot and root growth [29]. Uptake of cadmium by living cells causes many drastic changes, leading to cell death depending on the cadmium quantity and time duration of exposure [30]. However, in the present study, a similar decreasing trend in growth was observed in both lines at the 5th, 10th, and 15th day of the cadmium treatment.

A significant ($P \leq 0.001$) variation in leaf chlorophyll a and b, total chlorophyll, and carotenoid contents were recorded in both lines under cadmium stress both on the 5th, 10th, and 15th day of sampling (Tables 1 and 2). Overall, MTM-2 had more photosynthetic pigments than MTM-1 on the 5th, 10th, and 15th day of sampling under cadmium stress. However, the maximum reduction in chlorophyll a and b, total chlorophyll, and total carotenoids were observed at 9 and 12 mg $CdCl_2$ kg^{-1} in both lines, particularly on the 10th and 15th day of sampling in MTM-1.

The cadmium stress has been shown to enhance the stomatal closure and inhibit the photosynthesis via chlorophyll degradation in plants [31]. The cadmium damages the photosynthetic machinery of plants, particularly light harvesting complex-II and photosystem-I (PS-I) and PS-II [32]. Similarly, higher level of cadmium has been shown to reduce the synthesis of chlorophyll a, b and total chlorophyll contents in gram and sorghum [33, 34]. In line with previous studies, we found the maximum reduction in chlorophyll a, b contents at higher cadmium level (9 and 12 mg $CdCl_2$ kg^{-1}) in both lines, particularly on the 15th day of the treatment of cadmium. This implied that the light harvesting system was damaged based on the cadmium exposure time in maize. Plants possessing higher concentrations of chlorophyll and other accessory pigments tend to have higher shoot and root dry weights as these pigments are directly involved in the process of photosynthesis which is closely linked to plant growth and dry matter production [35]. In the present investigation, we observed a significant positive correlation of chlorophyll a ($r = 0.450^{***}$; 0.410^{***}), chlorophyll b ($r = 0.037$ ns; -0.03 ns), and carotenoids ($r = 0.259^{*}$; 0.265^{*}) with the shoot and root dry weights that was recorded and presented in Table 3.

Cadmium stress significantly altered leaf RMP, H2O2, MDA, and the total anthocyanin contents in both lines on 5th, 10th, and 15th day of sampling. Although leaf RMP, H2O2, MDA, and total anthocyanin contents increased significantly ($P \leq 0.001$) in both lines at all levels of cadmium stress, MTM-1 line had higher leaf RMP, H2O2, MDA, and total anthocyanin contents on the 5th, 10th, and 15th day of sampling, particularly at 12 mg $CdCl_2$ kg^{-1} on the 15th day of the sampling (Tables 1 and 2).

Cadmium has been shown to damage cell membranes, induce oxidative stress, and thus it enhances the ionic leakage in plants [36]. Cell membrane injury is associated with the production of reactive oxygen species (ROS), which indicated the production of MDA [3]. However, plants have both enzymatic and nonenzymatic antioxidant systems to hinder the production of ROS. Plants having the ability to synthesize these compounds are considered as tolerant under different abiotic stresses. Irrespective of the exposure time, in the present study, cadmium enhanced RMP, H2O2, and MDA contents in both lines. This indicated that the production of ROS is the major factor related to cadmium injury in maize plants. Cadmium-induced oxidative stress-mediated enhanced production of MDA contents has already been reported in maize and *Solanum* [36, 37]. When plants are exposed to abiotic stress, membrane integrity is greatly hampered [38]. Loss of membrane integrity is usually measured as a rise in cellular levels of MDA, a by-product of lipid peroxidation. Researchers have shown that MDA levels are negatively linked with plant growth [39]. Similarly in our study, MDA contents exhibited a negative correlation

Table 1: Mean sum of square from ANOVA of data for growth and biochemical attributes of two maize (*Zea mays* L.) lines under different treatments of cadmium after different time intervals.

S.O.V.	df	Shoot dry weight	Root dry weight	Leaf area	Chlorophyll a
Lines (*L*)	1	15.56***	3.97***	2074.52***	4.64***
Cadmium (Cd)	4	5.88***	2.97***	227.06***	5.68***
Time (*T*)	2	38.97***	13.64***	2109.14***	54.73***
L × Cd	4	1.33***	0.20***	53.27***	0.12***
L × *T*	2	0.61***	0.31***	2.85*	1.71***
Cd × *T*	8	0.21***	0.16***	1.69*	0.90***
L × Cd × *T*	8	0.44***	0.16***	18.06***	0.31***
Error	60	0.021	0.024	0.713	0.009
LSD 0.05	—	0.237	0.256	1.379	0.153
S.O.V.	df	Chlorophyll b	Total Chlorophyll	Carotenoids	RMP
Lines (*L*)	1	2.19***	13.26***	0.57***	366.91***
Cadmium (Cd)	4	10.97***	32.45***	0.77***	3665.94***
Time (*T*)	2	14.40***	112.56***	4.94***	14370.68***
L × Cd	4	0.13***	0.25***	0.01***	22.34***
L × *T*	2	0.12***	2.40***	0.16***	181.05***
Cd × *T*	8	0.77***	2.78***	0.12***	239.16***
L × Cd × *T*	8	0.10***	0.57***	0.04***	55.36***
Error	60	0.011	0.020	0.00026	2.578
LSD 0.05	—	0.171	0.233	0.026	2.622
S.O.V.	df	MDA	H2O2	TAA	Free proline
Lines (*L*)	1	554.23***	117.31***	17.90***	102.86***
Cadmium (Cd)	4	270.68***	310.40***	3.93***	86.99***
Time (*T*)	2	651.96***	834.04***	6.84***	89.23***
L × Cd	4	42.74***	3.53***	0.77***	1.46***
L × *T*	2	79.58***	105.45***	4.81***	6.63***
Cd × *T*	8	3.69**	6.84***	0.07***	2.95***
L × Cd × *T*	8	6.78***	7.03***	0.07***	0.31***
Error	60	1.030	0.143	0.017	0.061
LSD 0.05	—	1.657	0.618	0.211	0.403
S.O.V.	df	Anthocyanin			
Lines (*L*)	1	101.98***			
Cadmium (Cd)	4	351.97***			
Time (*T*)	2	193.48***			
L × Cd	4	8.04***			
L × *T*	2	9.26***			
Cd × *T*	8	3.16***			
L × Cd × *T*	8	8.35***			
Error	60	0.254			
LSD 0.05	—	0.824			

*,**,***: significant at 0.05, 0.01, and 0.001 levels, respectively; ns: nonsignificant.
RMP: relative membrane permeability; MDA: malendialdehyde; H2O2: hydrogen peroxide;
TAA: total free amino acids.

($r = -300^{**}$; -0.306^{**}) with shoot and root dry weights, respectively, presented in Table 3. In addition, the production of reactive oxygen species is also a great threat to plant growth. ROS are known for inducing substantial damage to pigments as is evident from the present investigation, where H2O2 exhibited a negative correlation ($r = -0.744^{***}$; -0.817^{***}, -0.846^{***}) with chlorophyll a, b and carotenoids, respectively, presented in Table 3.

H2O2 is also known to inhibit the Calvin cycle that ultimately results in reduced photosynthetic rates. This could have been the major factor for negative correlation of H2O2 with the shoot ($r = -0.502^{***}$) and root ($r = -0.236^{*}$) dry weights presented in Table 3. Measures of relative membrane permeability (RMP) in plants exposed to various environmental stresses including cadmium stress are taken as indicators for stress-induced damage in plants. Plants under

Table 2: Means comparison data for growth and biochemical attributes of two maize (*Zea mays* L.) lines under different treatments of cadmium after different time intervals.

Lines	Time (d)	Cd ($mg\,kg^{-1}$)	SDW	RDW	Leaf area	Chl a	Chl b	Tot-Chl	Car.	RMP	MDA	H2O2	TAA	Proline	Anth.
MTM-1	5	0	2.23 ± 0.01^{no}	2.23 ± 0.04^{kl}	20.47 ± 0.06^{op}	3.61 ± 0.22^{c}	2.57 ± 0.26^{c}	6.18 ± 0.35^{b}	1.36 ± 0.00^{a}	32.72 ± 0.33^{n}	8.04 ± 0.13^{tu}	6.14 ± 0.01^{s}	0.97 ± 0.02^{lmno}	5.99 ± 0.06^{j}	2.44 ± 0.03^{o}
		3	2.08 ± 0.06^{o}	1.81 ± 0.06^{no}	19.15 ± 1.47^{p}	3.20 ± 0.08^{d}	2.38 ± 0.05^{de}	5.58 ± 0.10^{c}	1.20 ± 0.02^{b}	35.39 ± 0.67^{n}	9.46 ± 0.06^{st}	8.20 ± 0.06^{q}	1.12 ± 0.01^{jklm}	6.20 ± 0.04^{j}	6.62 ± 0.28^{k}
		6	1.73 ± 0.04^{pq}	1.41 ± 0.05^{pq}	17.68 ± 0.09^{q}	2.71 ± 0.04^{e}	2.09 ± 0.04^{f}	4.79 ± 0.07^{de}	0.97 ± 0.00^{d}	39.40 ± 0.33^{n}	12.66 ± 0.32^{qr}	11.45 ± 0.09^{no}	1.17 ± 0.00^{jkl}	7.77 ± 0.04^{g}	9.13 ± 0.28^{i}
		9	1.56 ± 0.02^{q}	1.33 ± 0.04^{qr}	16.67 ± 0.81^{qr}	2.10 ± 0.06^{f}	1.19 ± 0.03^{i}	3.29 ± 0.07^{g}	0.78 ± 0.01^{f}	45.06 ± 1.00^{l}	15.93 ± 0.59^{klmn}	13.46 ± 0.26^{lm}	1.42 ± 0.16^{hi}	9.60 ± 0.19^{d}	10.70 ± 0.15^{gh}
		12	1.30 ± 0.03^{r}	1.13 ± 0.06^{r}	15.70 ± 0.32^{r}	0.55 ± 0.04^{klm}	0.41 ± 0.17^{op}	0.96 ± 0.13^{mn}	0.56 ± 0.01^{h}	56.39 ± 0.33^{j}	18.90 ± 0.57^{gh}	16.14 ± 0.16^{gh}	1.61 ± 0.09^{gh}	12.57 ± 0.22^{b}	13.30 ± 0.28^{e}
	10	0	4.76 ± 0.02^{cd}	2.82 ± 0.08^{fg}	37.27 ± 0.43^{e}	1.63 ± 0.08^{g}	3.03 ± 0.01^{b}	4.66 ± 0.09^{e}	0.85 ± 0.02^{e}	32.55 ± 0.64^{n}	8.21 ± 0.39^{tu}	6.87 ± 0.00^{r}	0.87 ± 0.05^{no}	4.05 ± 0.16^{n}	4.12 ± 0.28^{lm}
		3	3.95 ± 0.10^{f}	2.68 ± 0.05^{ghi}	34.74 ± 0.11^{hi}	1.02 ± 0.00^{i}	2.36 ± 0.03^{de}	3.38 ± 0.03^{fg}	0.65 ± 0.01^{g}	46.93 ± 1.20^{l}	17.94 ± 0.52^{ghij}	11.85 ± 0.06^{n}	1.00 ± 0.04^{lmno}	4.55 ± 0.01^{m}	9.13 ± 0.28^{i}
		6	3.42 ± 0.02^{g}	2.61 ± 0.05^{ghi}	33.46 ± 0.33^{ij}	0.65 ± 0.02^{k}	1.08 ± 0.02^{ij}	1.73 ± 0.04^{j}	0.38 ± 0.01^{k}	67.24 ± 0.56^{i}	19.40 ± 1.20^{fg}	12.87 ± 0.34^{m}	1.31 ± 0.11^{ijk}	5.51 ± 0.48^{k}	10.24 ± 0.09^{gh}
		9	3.05 ± 0.08^{hi}	2.50 ± 0.23^{hijk}	32.53 ± 0.50^{jk}	0.47 ± 0.02^{lm}	0.91 ± 0.04^{jk}	1.38 ± 0.02^{k}	0.31 ± 0.01^{l}	83.60 ± 1.19^{de}	22.13 ± 1.37^{e}	15.04 ± 0.13^{j}	1.36 ± 0.05^{ij}	6.91 ± 0.02^{hi}	15.25 ± 0.02^{d}
		12	2.88 ± 0.13^{ijk}	2.47 ± 0.06^{ijk}	31.96 ± 0.03^{k}	0.27 ± 0.01^{o}	0.21 ± 0.00^{qr}	0.48 ± 0.02^{pq}	0.12 ± 0.00^{o}	91.58 ± 1.35^{b}	26.03 ± 0.80^{cd}	17.63 ± 0.15^{f}	1.51 ± 0.09^{hi}	8.93 ± 0.03^{ef}	17.76 ± 0.28^{b}
	15	0	4.78 ± 0.09^{cd}	3.58 ± 0.08^{bc}	36.96 ± 0.35^{ef}	0.81 ± 0.03^{j}	1.25 ± 0.03^{i}	2.06 ± 0.02^{hi}	0.30 ± 0.01^{l}	66.13 ± 1.73^{i}	16.81 ± 0.16^{ijkl}	15.86 ± 0.10^{hi}	0.80 ± 0.08^{o}	3.70 ± 0.17^{n}	11.07 ± 0.74^{fg}
		3	4.28 ± 0.07^{e}	3.40 ± 0.16^{cd}	35.47 ± 0.38^{fgh}	0.25 ± 0.01^{o}	0.69 ± 0.03^{lm}	0.95 ± 0.04^{mn}	0.14 ± 0.01^{o}	80.85 ± 0.36^{fg}	24.58 ± 0.71^{d}	21.51 ± 0.37^{d}	0.90 ± 0.06^{mno}	4.09 ± 0.01^{n}	11.91 ± 0.28^{f}
		6	3.10 ± 0.10^{hi}	2.88 ± 0.05^{fg}	30.04 ± 0.16^{l}	0.18 ± 0.02^{o}	0.36 ± 0.01^{opq}	0.54 ± 0.02^{op}	0.08 ± 0.00^{p}	84.88 ± 1.32^{de}	27.39 ± 0.25^{bc}	26.19 ± 0.55^{b}	0.97 ± 0.03^{lmno}	4.96 ± 0.02^{lm}	13.86 ± 0.48^{e}
		9	3.01 ± 0.07^{hij}	2.38 ± 0.09^{jk}	29.28 ± 0.33^{l}	0.15 ± 0.01^{o}	0.26 ± 0.01^{pqr}	0.41 ± 0.01^{pq}	0.07 ± 0.00^{pqr}	93.24 ± 1.54^{b}	28.34 ± 0.56^{b}	28.05 ± 0.25^{a}	1.29 ± 0.07^{ijk}	5.18 ± 0.09^{kl}	16.42 ± 0.36^{c}
		12	2.49 ± 0.11^{lm}	1.95 ± 0.09^{mn}	25.23 ± 0.16^{n}	0.12 ± 0.01^{o}	0.16 ± 0.01^{r}	0.28 ± 0.01^{q}	0.06 ± 0.00^{pqr}	99.72 ± 1.54^{a}	30.47 ± 1.20^{a}	28.33 ± 0.04^{a}	1.48 ± 0.06^{hi}	6.67 ± 0.15^{i}	19.43 ± 0.28^{a}

Table 2: Continued.

Lines	Time (d)	Cd (mg kg^{-1})	SDW	RDW	Leaf area	Chl a	Chl b	Tot-Chl	Car.	RMP	MDA	H2O2	TAA	Proline	Anth.
MTM-2	5	0	2.70 ± 0.11^{kl}	2.22 ± 0.06^{klm}	30.43 ± 0.35^{l}	4.42 ± 0.10^{a}	3.20 ± 0.01^{ab}	7.63 ± 0.11^{a}	1.34 ± 0.01^{a}	23.74 ± 0.33^{p}	7.59 ± 0.30^{u}	3.44 ± 0.05^{t}	2.05 ± 0.13^{f}	6.86 ± 0.03^{i}	2.73 ± 0.28^{o}
		3	2.78 ± 0.01^{jk}	2.48 ± 0.07^{ijk}	35.41 ± 0.34^{gh}	3.92 ± 0.04^{b}	2.49 ± 0.01^{cd}	6.41 ± 0.06^{b}	1.13 ± 0.02^{c}	29.06 ± 0.58^{o}	9.29 ± 0.30^{stu}	7.45 ± 0.03^{r}	2.86 ± 0.04^{d}	7.91 ± 0.02^{g}	4.80 ± 0.13^{l}
		6	2.38 ± 0.09^{mn}	2.03 ± 0.01^{lmn}	27.41 ± 0.57^{m}	3.13 ± 0.02^{d}	2.27 ± 0.03^{e}	5.40 ± 0.04^{c}	0.75 ± 0.01^{f}	33.40 ± 0.88^{m}	10.45 ± 0.13^{s}	10.46 ± 0.30^{p}	3.13 ± 0.12^{c}	9.17 ± 0.06^{e}	7.84 ± 0.54^{j}
		9	2.16 ± 0.09^{no}	1.90 ± 0.03^{n}	25.41 ± 0.37^{n}	3.10 ± 0.00^{d}	1.76 ± 0.03^{g}	4.86 ± 0.03^{de}	0.49 ± 0.01^{i}	40.07 ± 0.58^{m}	12.47 ± 0.54^{r}	13.68 ± 0.25^{kl}	3.48 ± 0.06^{b}	11.21 ± 0.11^{c}	10.68 ± 0.16^{gh}
		12	1.83 ± 0.08^{p}	1.61 ± 0.06^{op}	21.37 ± 0.28^{o}	2.61 ± 0.03^{e}	0.86 ± 0.00^{kl}	3.47 ± 0.04^{fg}	0.43 ± 0.01^{j}	47.44 ± 1.84^{l}	14.58 ± 0.35^{mnop}	15.44 ± 0.08^{ij}	3.79 ± 0.09^{a}	14.67 ± 0.13^{a}	11.78 ± 0.13^{f}
	10	0	4.42 ± 0.12^{e}	3.00 ± 0.08^{ef}	42.94 ± 0.55^{c}	1.76 ± 0.06^{g}	3.23 ± 0.02^{a}	4.99 ± 0.08^{d}	0.26 ± 0.01^{m}	47.39 ± 1.18^{l}	12.20 ± 0.36^{r}	10.91 ± 0.27^{op}	0.87 ± 0.03^{no}	6.85 ± 0.03^{i}	3.02 ± 0.01^{no}
		3	5.56 ± 0.09^{a}	3.31 ± 0.09^{cd}	51.12 ± 0.98^{a}	1.33 ± 0.01^{h}	2.27 ± 0.00^{e}	3.60 ± 0.02^{f}	0.19 ± 0.00^{n}	53.07 ± 1.41^{k}	13.20 ± 0.31$_{pqr}$	12.02 ± 0.02^{n}	1.08 ± 0.05^{klmn}	7.30 ± 0.08^{h}	6.07 ± 0.56^{k}
		6	4.75 ± 0.06^{cd}	3.20 ± 0.06^{de}	45.04 ± 0.58^{b}	1.29 ± 0.03^{h}	2.08 ± 0.02^{f}	3.37 ± 0.02^{fg}	0.13 ± 0.01^{o}	65.12 ± 0.57^{i}	14.33 ± 0.17^{nopq}	13.49 ± 0.07^{lm}	1.77 ± 0.04^{g}	8.65 ± 0.13^{f}	9.96 ± 0.04^{hi}
		9	3.98 ± 0.08^{f}	2.50 ± 0.03^{hijk}	39.43 ± 0.32^{d}	0.62 ± 0.02^{kl}	1.25 ± 0.02^{i}	1.87 ± 0.03^{ij}	0.12 ± 0.01^{o}	78.65 ± 0.80^{g}	15.25 ± 0.54^{lmno}	14.96 ± 0.03^{j}	2.47 ± 0.10^{e}	11.03 ± 0.02^{c}	11.74 ± 0.08^{f}
		12	3.16 ± 0.09^{h}	2.06 ± 0.01^{lmn}	35.95 ± 0.57^{efgh}	0.19 ± 0.00^{o}	0.67 ± 0.03^{m}	0.86 ± 0.03^{mn}	0.05 ± 0.00^{qr}	85.83 ± 0.29^{cd}	17.47 ± 0.76^{hijk}	16.10 ± 0.56^{gh}	2.77 ± 0.13^{d}	12.23 ± 0.34^{b}	13.02 ± 0.05^{e}
	15	0	4.38 ± 0.09^{e}	3.75 ± 0.10^{ab}	39.65 ± 0.34^{d}	0.64 ± 0.01^{k}	1.47 ± 0.03^{h}	2.12 ± 0.03^{h}	0.25 ± 0.01^{m}	56.16 ± 0.36^{j}	13.99 ± 0.23^{opqr}	11.71 ± 0.24^{n}	0.76 ± 0.04^{o}	4.72 ± 0.11^{m}	3.76 ± 0.05^{mn}
		3	5.32 ± 0.04^{b}	3.98 ± 0.24^{a}	45.70 ± 0.08^{b}	0.50 ± 0.01^{klm}	0.77 ± 0.01^{klm}	1.27 ± 0.00^{kl}	0.14 ± 0.00^{o}	72.17 ± 0.61^{h}	15.37 ± 0.27^{lmno}	14.15 ± 0.08^{k}	0.97 ± 0.03^{lmno}	4.88 ± 0.06^{lm}	6.07 ± 0.28^{k}
		6	4.98 ± 0.14^{c}	3.36 ± 0.05^{cd}	44.67 ± 0.33^{b}	0.44 ± 0.01^{mn}	0.61 ± 0.01^{mn}	1.06 ± 0.01^{lm}	0.07 ± 0.00^{pq}	82.48 ± 0.88^{ef}	16.32 ± 0.27^{jklm}	16.74 ± 0.19^{g}	1.28 ± 0.05^{ijk}	6.07 ± 0.03^{j}	14.97 ± 0.28^{d}
		9	4.66 ± 0.14^{d}	3.30 ± 0.08^{cd}	39.33 ± 0.33^{d}	0.29 ± 0.01^{no}	0.48 ± 0.00^{no}	0.78 ± 0.01^{no}	0.07 ± 0.00^{pqr}	87.71 ± 0.33^{c}	18.37 ± 0.33^{ghi}	20.08 ± 0.06^{e}	1.83 ± 0.01^{g}	7.91 ± 0.04$_{g}$	15.53 ± 0.10^{d}
		12	4.03 ± 0.09^{f}	2.78 ± 0.07^{fgh}	36.77 ± 0.24^{efg}	0.27 ± 0.01^{o}	0.22 ± 0.01^{qr}	0.49 ± 0.00^{pq}	0.04 ± 0.00^{r}	92.82 ± 0.66^{b}	20.97 ± 0.02^{ef}	24.72 ± 0.03^{c}	2.05 ± 0.04^{f}	9.29 ± 0.09^{de}	17.47 ± 0.28^{b}

All the values represent mean + S.E. and values that show the same superscript letter in the same column are not significantly different at $P \leq 0.05$.
SFW: shoot fresh weight; RDW: root dry weight; Chl. a: chlorophyll a; Chl. b: chlorophyll b; Car.: carotenoids; Anth.: anthocyanin.
MDA: malendialdehyde; RMP: relative membrane permeability; H2O2: hydrogen peroxide; TAA: total free amino acids.

Table 3: Correlation among growth and biochemical attributes of two maize (*Zea mays* L.) lines under different treatments of cadmium after different time intervals.

Attributes	SDW	RDW	LA	Chl. a	Chl. b	Car	RMP	MDA	H2O2	TAA	Proline	Anth.
SDW	1											
RDW	0.908^{***}	1										
LA	0.938^{***}	0.852^{***}	1									
Chl a	-0.450^{***}	-0.410^{***}	-0.390^{***}	1								
Chl b	0.037 ns	−0.031 ns	0.057 ns	0.748^{***}	1							
Car.	-0.259^{*}	-0.265^{*}	−0.213 ns	0.953^{***}	0.913^{***}	1						
RMP	-0.506^{***}	-0.465^{***}	-0.531^{***}	-0.873^{***}	-0.702^{***}	-0.854^{***}	1					
MDA	-0.300^{**}	-0.306^{**}	-0.298^{***}	-0.886^{***}	-0.853^{***}	-0.931^{***}	-0.862^{***}	1				
H2O2	-0.502^{***}	-0.236^{**}	−0.012 ns	-0.774^{***}	-0.817^{***}	-0.846^{***}	-0.690^{***}	0.855^{***}	1			
TAA	0.062 ns	0.094 ns	0.023 ns	-0.742^{***}	-0.845^{***}	-0.839^{***}	-0.758^{***}	0.863^{***}	0.902^{***}	1		
Proline	-0.397^{***}	-0.432^{***}	−0.203 ns	-0.361^{***}	−0.041 ns	0.203 ns	0.058 ns	−0.151 ns	−0.194 ns	−0.056 ns	1	
Anth.	-0.490^{***}	-0.602^{***}	-0.329^{**}	0.162 ns	−0.184 ns	0.016 ns	−0.044 ns	−0.042 ns	−0.100 ns	0.008 ns	0.786^{***}	1

*,**,***: significant at 0.05, 0.01, and 0.001 levels, respectively; ns: nonsignificant.
SFW: shoot fresh weight; RDW: root dry weight; Chl. a: chlorophyll a; Chl. b: chlorophyll b; Car.: carotenoids; Anth.: anthocyanin.
MDA: malendialdehyde; RMP: relative membrane permeability; H2O2: hydrogen peroxide; TAA: total free amino acids.

cadmium stress exhibit increase in the permeability of membranes that ultimately leads to loss of membrane integrity. Researchers take the ability of plasma membranes to control the movement of ions across the cell as a potential selection criterion to measure the extent of damage to a great variety of tissues [40]. Likewise in the present investigation, we have observed a negative association of RMP ($r = -0.506^{***}$, -0.465^{***}, -0.531^{**}, -0.873^{***}, -0.702^{***}, -0.854^{***}) with shoot and root dry weights, leaf area, chlorophyll a, b, and carotenoids, respectively, presented in Table 3. This could have been due to the inability of plasma membrane to control the movement of ions across the cell.

Leaf free proline, total free amino acid, and anthocyanin contents significantly ($P \leq 0.001$) differed in both lines under cadmium stress. The plants of MTM-2 line accumulated more free proline, total free amino acid, and anthocyanin contents as compared with the MTM-1 on the 5th, 10th, and 15th day of sampling under cadmium stress (Tables 1 and 2). Moreover higher free proline and total free amino acid contents were produced on the 5th sampling in both lines, while production of free proline and total free amino acids was decreasing on the 10th and 15th day of sampling at all levels of cadmium stress. Furthermore, total anthocyanin contents increased gradually on the 5th, 10th, and 15th day of sampling at all levels of cadmium stress in both lines.

Free proline accumulation occurred in cadmium-treated plants of MTM-2 line. Thus, the accumulation of proline in response to cadmium stress was due to the production of ROS. Previous studies have shown those plants which produced more proline and free amino acids due to more production of hydroxyl radicals. Proline is the only molecule that protects plants against singlet oxygen and free radical since proline acts as a singlet oxygen quencher and as a scavenger of OH radicals [41, 42]. Thus, proline is not only an important molecule in redox signalling but also an effective quencher of reactive oxygen species formed in all plants against abiotic stress while in others proline was produced as an indication of stress [43]. Similarly, in our investigation, we have recorded that proline did not contribute to a great extent in conferring tolerance to plants against cadmium stress. This could have been due to the fact that endogenous levels of proline were negatively correlated ($r = -0.397^{***}$, -0.432^{***}, -0.361^{***}, −0.194 ns) to shoot and root dry weights, chlorophyll a, and H2O2 (Table 3). In some reports, it has been shown that proline stabilizes the subcellular compartments of cells [44] which were not the case in the present investigation.

There are many reports which show that anthocyanins contents are able to quench the oxygen radicals [45]. In the present investigation, a significant increase in anthocyanin contents was observed in both maize lines, being the highest in MTM-1 line at the level of cadmium stress. Our results of anthocyanins contents are similar to some earlier reports of increases in endogenous levels of flavonoids and total anthocyanins under abiotic stress [46]. Furthermore, flavonoids act as nonenzymatic antioxidants and protect the plants against ROS-induced oxidative stress. Moreover, loss of membrane integrity in terms of lipid peroxidation has been reported in anthocyanin deficient mutant of *Arabidopsis* [47]. Therefore, anthocyanins are considered as a defensive yardstick against various abiotic stresses in plants [48]. Thus, anthocyanin contents may affect the stress defense mechanism.

4. Conclusions

Irrespective of exposure time, cadmium stress affected morphophysiological attributes in both lines. However, on the 15th day of treatment, the cadmium effects and the response of lines to cadmium stress became more evident. The cadmium stress produced oxidative stress in the plants of both lines which was evident from the increased synthesis of H2O2 and MDA contents and increased RMP. Taken together, the results suggested that cadmium-induced oxidative stress was the main cause of reduced plant growth in both lines. Based on having more accumulation of free amino acids, free proline and total anthocyanin contents at all cadmium levels,

and the stability or enhancement in plant biomass and leaf area particularly occurred at 3 mg $CdCl_2$ kg^{-1} concentration, the line MTM-2 was considered tolerant as compared with MTM-1 line.

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